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Identification, Purification, and Characterization of a Calcium-dependent Endonuclease (NUC18) from Apoptotic Rat Thymocytes

NUC18 IS NOT HISTONE H_2B*

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Glucocorticoids stimulate apoptosis in rat thymocytes that is characterized by internucleosomal DNA degradation. We have previously identified an 18-kDa calcium-dependent nuclease whose activity is associated with this DNA degradation. The existence of this nuclease has been challenged by Alnemri and Litwack (1989) *J. Biol. Chem.* 264, 4104–4111, who suggest that the nuclease we observed was histone H_2B. We report here a modified nuclease assay which uses [^{32}P] DNA as a substrate that has enabled the purification and characterization of the 18-kDa nuclease (NUC18). Using Bio-Rex 70 chromatography in conjunction with this assay, we show that NUC18 can be separated from histone H_2B. Enzymatically active NUC18, purified to apparent homogeneity, failed to react with two different anti-histone H_2B antibodies. NUC18 was inactive in the absence of calcium and known inhibitors of apoptosis, *i.e.* zinc and aurintricarboxylic acid inhibit its activity. Although NUC18 activity was detected in nuclear extracts of thymocytes of both control and glucocorticoid-treated thymocytes, these activities were distinct. Gel filtration analysis revealed that NUC18 was present as a high molecular weight complex (>100 kDa) in both groups of cells, whereas it also existed as a low molecular weight form in glucocorticoid-treated cells. Thus, NUC18 remains a candidate for the endonuclease responsible for the DNA degradation component of the apoptotic process.

Glucocorticoids have profound effects on thymic lymphocytes that ultimately lead to lymphocytolysis (2–5). Numerous metabolic functions of thymocytes are rapidly altered in response to glucocorticoid treatment. These responses include altered uptake of glucose and other metabolic precursors (6), inhibition of RNA and protein synthesis (7, 8), decreased ATP production (9), and enhanced catabolism of both protein and RNA (10, 11). The most profound effects, however, seem to be on DNA where glucocorticoids are known to promote DNA degradation (2–5, 14).

It is now well recognized that glucocorticoid-induced lymphocytolysis in the thymus exhibits all of the morphological and biochemical changes that characterize apoptosis, or programmed cell death (2–4, 12, 13). Programmed cell death is an important physiological process that occurs in a wide range of biological processes including embryogenesis, differentiation, metamorphosis, and normal and neoplastic tissue growth (15–20). Accordingly, there are many physiological signals that can stimulate apoptosis. For example, programmed cell death occurs in the prostate following androgen withdrawal (21), in cytotoxic T-lymphocytes in the absence of interleukin-2 (22), in sympathetic neurons deprived of nerve growth factor (23), in lactating mammary tissue following weaning due to decreased prolactin (24), and in lymphoid tissue in response to glucocorticoids (3, 5, 25). Although these and many other signals can initiate programmed cell death in various cell types, the cascade of events that subsequently takes place appears to be similar in most instances (15–20). Morphologically, cells that are committed to die undergo several dramatic changes (17, 18), including development of pyknotic nuclei, extensive chromatin condensation, and a loss of cell volume. This shrinkage is associated with membrane convolution and the formation of blebs on the cell surface, which permits the dying cells to be recognized and removed by nearby phagocytic cells. Associated with chromatin condensation is the action of a nuclease activity that cleaves chromatin in the linker regions between nucleosomes (3, 4, 14, 26, 27, 44). This results in the generation of a series of DNA fragments appearing in 180–200 base pair increments. This internucleosomal DNA degradation can be blocked by inhibitors of transcription and translation (25, 29, 30); hence, apoptotic cells appear to be responsible for their own death.

As mentioned above, one of the hallmarks of programmed cell death is internucleosomal DNA degradation. This phenomenon has been most extensively characterized in lymphoid tissue following glucocorticoid treatment (3–5, 17, 28). Internucleosomally cleaved DNA is detectable in the thymus shortly after glucocorticoid administration (25, 28), prior to alterations in cell viability (3, 25, 28, 30). The killing of thymocytes by glucocorticoids appears to occur by a glucocorticoid receptor-dependent mechanism since this response is specific to glucocorticoids and since glucocorticoid antagonists such as RU486 prevent DNA degradation and other characteristics of apoptosis (25).

We have focused our efforts on the early events that mediate DNA degradation and the role of DNA degradation in programmed cell death. We have previously identified a low molecular weight calcium-dependent nuclease(s) that may play a role in apoptosis (31). However, the existence of this nuclease has been challenged by Alnemri and Litwack (1), who demonstrate that histones can express artifactual nuclease activity in nuclease activity gels due to the ability of histones to block ethidium bromide intercalation into DNA. This data, coupled with the fact that they were unable to

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purify calcium-dependent endonuclease activity in the CEM-C7 cells used for their study led Alnemri and Litwack to conclude that the nuclease activity we observed in thymus must have been due to histone contamination. However, these authors have also reported that the lymphoid-derived CEM-7 cell line that they studied does not respond to glucocorticoids by the same mechanism as rat thymocytes, and they have recently acknowledged that the CEM-C7 cells do not contain the calcium-dependent endonuclease (32). Hence, it is predictable that Alnemri and Litwack were unable to detect the nuclease activity since it does not appear to be present in CEM-C7 cells. We report here on the use of a modified nuclease assay, which allows the direct identification of nuclear extract proteins that possess nuclease activity. This assay does not rely on ethidium bromide staining; rather, it employs radiolabeled DNA as the substrate. This modification circumvents the problem of detecting false positive signals by ethidium bromide staining, since a loss of the radioactive signal is due to actual DNA loss and not due to protein quenching of ethidium bromide intercalation. The resulting autoradiograph confirms the presence of "holes" in the gel that correspond to nuclease proteins. Using this modified nuclease assay, we have localized nuclease activity from rat thymocytes to a distinct 18-kDa protein. We have proceeded to purify this protein and to characterize its enzymatic properties with respect to pH optimum, divalent cation requirements, and sensitivity to inhibitors. Furthermore we show that histone H_2B can be effectively removed in a single chromatography step, as confirmed by two different antihistone H_2B antisera, without causing any apparent loss in nuclease activity. Finally, biochemical studies show that although control thymocytes and glucocorticoid-treated cells have equivalent amounts of nuclease activity, the native size of the NUC18 protein and/or protein complex apparently changes in response to steroid.

MATERIALS AND METHODS

Preparation of Thymocyte Nuclear Extract—Nuclear extracts were prepared as described by Compton and Cidlowski (31) with several important modifications. Briefly, male Sprague-Dawley rats (85–100 g), obtained from either Charles River Laboratories, Inc. (Wilmington, MA) or Dominion Laboratories (Dublin, VA), were bilaterally adrenalectomized and maintained on 0.85% NaCl and rat chow *ad libitum*. Within 5–10 days after surgery, dexamethasone (Steraloids, Inc., Wilton, NH), 5 mg/kg of body weight, suspended in phosphate-buffered saline or phosphate-buffered saline alone, was administered intraperitoneally. Thymi were harvested 5 h after injection, and thymocytes were prepared by homogenization in ice-cold phosphate-buffered saline, followed by two Nitex mesh (202 mm) filtrations. Thymocytes were lysed in ice-cold hypotonic buffer (10 mM MgCl) containing 0.25% Nonidet P-40. Nuclei were collected by centrifugation and extracted in 1.5 ml/thymus in 0.4 M NaCl, 20 mM Tris-HCl, pH 7.5, 1 mM EDTA at 4 °C for 1 h. Chromatin was removed by a 1-h ultracentrifugation step at 110,000 × *g*. Extracts were stored at −20 °C. The dialysis step previously employed at this stage was omitted.

SDS-PAGE[1]—Standard Laemmli gels (33) were used for protein electrophoresis. Protein concentrations were determined by the method of Bradford (34), and protein aliquots were heated for 10 min at 60 °C in SDS sample buffer (10% glycerol, 2.3% SDS, 0.63 M Tris-HCl, pH 8.5, 5 mM dithiothreitol) immediately before loading into the gel. The SDS used for all gels was GIBCO-BRL Ultrapure (Gaithersburg, MD) that was recrystallized twice (47). Protein samples were electrophoresed using a Hoefer Scientific Instruments (San Francisco, CA) Tall Mighty Small vertical slab gel (1.5 mm × 8 cm × 11 cm) consisting of a 4% stacking gel, pH 8.5, and a 15% separating gel, pH 6.8. Gels were run under constant current at 150–175 V for 1.5–2 h. Gels were stained overnight in 0.025% Coomassie Brilliant Blue R-250 and destained for 6–8 h in 16% methanol, 5% acetic acid. For gels requiring silver stain detection, Coomassie Blue-stained gels were further destained in 50% methanol with two washes, equilibrated in 20% ethanol, stained 15 min in 0.8% silver nitrate dissolved in 0.8% NaOH, 1.5% NH_4OH, rinsed with deionized water, and developed in 0.01% citric acid, 0.04% formaldehyde. Reactions were stopped using destain solution, and the gels were photographed.

Protein Purification—All chromatographic separations were performed at 4 °C, and all buffers contained 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, and the specified concentrations of NaCl (ranging from 10 mM to 1.5 M). All columns were packed by gravity flow and then equilibrated with starting buffer at the specified flow rate. Flow rates for three of the columns (Bio-Rex 70, DEAE-Bio-Gel, and phenyl-Sepharose) were kept at approximately 40 ml/h with a Pharmacia LKB Biotechnology Inc. peristaltic pump. The flow rate for the gel filtration column, Bio-Gel P-100, was approximately 10 ml/h. UV absorbance of throughput was monitored by an Isco (Lincoln, NE) model 226 absorbance monitor at 280 nm and recorded on an Isco model 615A recorder. Nuclear extracts (in 0.4 M NaCl) were prepared from 25 rat thymi (in approximately 40 ml) and passed over a 30-ml Bio-Rex 70 column (Bio-Rad), and the flow through was collected. The flow-through (containing approximately 85% of the total protein in 75 ml) was dialyzed into 10 mM NaCl, 20 mM Tris-HCl, pH 7.5, 1 mM EDTA and passed over a 20-ml DEAE-Bio-Gel A column (Bio-Rad) and the flow-through was collected. The flow-through (250 ml, containing approximately 2.5% of the total extract protein) was adjusted to 1.5 M NaCl and chromatographed on a 20-ml phenyl-Sepharose CL-4B column (Pharmacia/LKB). The nuclease activity was eluted with 0.5 M NaCl. This fraction (30 ml, containing 1.9% of total protein) was further concentrated in a Centricell 10,000 (Polysciences, Inc., Warrington, PA) to 2 ml. The concentrated phenyl-Sepharose eluate was separated by preparative SDS-PAGE using standard Laemmli techniques. The preparative gels (Hoefer, 1.5 mm × 14 cm × 16-cm vertical slab gels) were run as described above, with two modifications. The 15% separating gels were polymerized overnight, and 0.1 mM thioglycolic acid was included in the upper gel running buffer to prevent destruction of certain amino acids by free radicals or oxidants trapped in the gel matrix (47). Gels were electrophoresed for 4–5 h at 150–175 V. After electrophoresis, the gel was soaked in transfer buffer (10 mM CAPS (Sigma), pH 11, 10% methanol) and then electroblotted onto a polyvinylidene difluoride membrane (Immobilon P, Millipore Corp., Bedford, MA) in transfer buffer at 0.5 A for 2.5 h at 4 °C. Transferred proteins were identified by staining with the reversible stain Ponceau S (Sigma). Gels were stained in 0.5% Ponceau S in 1.0% acetic acid for 2 min and destained in water for 1 min. The protein band at 18 kDa was excised, and the protein was eluted using 1.0% Triton X-100, 0.5% SDS by incubation for 1 h at room temperature. The eluted protein was stored at −20 °C. The yield of purified NUC18 protein from four such purifications ranged from 2 to 5 μg.

Nuclease Activity Assay—To identify and characterize the 18-kDa nuclease, we have used the nuclease activity gel system originally described by Rosenthal and Lacks (43) with several modifications. All activity gels were standard Laemmli gels as described above using the Hoefer Tall Mighty Small (1.5 mm × 8 cm × 11 cm) vertical apparatus. 1 mg/ml heat-denatured calf thymus DNA (Sigma), along with 750,000 cpm of nick-translated plasmid DNA (nick translation kit from Boehringer-Mannheim), were incorporated directly into the 15% separating gel matrix during polymerization. Protein samples were heated to 60 °C for 10 min in sample buffer and electrophoresed at 175 V for 1.5 h. Micrococcal nuclease (Worthington; 25,882 units/mg of protein) was run as a positive control on every gel. After electrophoresis, the gels were washed overnight in TEM buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 2 mM $MgCl_2$) to remove SDS, thereby allowing protein renaturation. Gels were stained in TEM buffer containing 1 μg/ml ethidium bromide (Sigma) for 30 min at 37 °C and photographed on a 302 nm transilluminator. To activate nucleases, 2 mM calcium chloride was added to the buffer, and the gel was incubated at 37 °C for 3 h with shaking. Gel treatments requiring other cations or inhibitors in the buffer were incubated for 1 h at 37 °C with their respective buffers prior to the addition of calcium. During the 3-h incubation with calcium, the gels were periodically checked by UV transillumination to localize areas and extent of apparent DNA loss. The gels were subsequently washed in TEM buffer containing 10% glycerol for 20 min, dried under vacuum for 2 h at 60 °C, and processed for autoradiography overnight at −70 °C.

[1] The abbreviations used are: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; CAPS, 3-(cyclohexylamino)propanesulfonic acid.

Western Blotting—Gels to be processed for Western analysis were electrophoresed as described above and then soaked in transfer buffer (10 mM CAPS, pH 11, and 10% methanol) to remove Tris and glycine. Proteins were then electroblotted onto polyvinylidene difluoride membranes (Immobilon P, Millipore Corp.) in transfer buffer at 60 V for 3 h at 4 °C. Membranes were not allowed to dry and were blocked in Blotto (10% Carnation nonfat milk, 0.15 M NaCl, 10 mM Tris-HCl, pH 7.4, 0.05% Tween 20) for 4 h at room temperature. Blots were incubated with primary antibody overnight at 4 °C in Blotto, washed with Blotto (3 × 5 min at room temperature) and hybridized with ^{125}I-Protein A (ICN Biomedicals, Costa Mesa, CA) (5 μCi/ml Blotto) for 2 h at room temperature. Subsequent washes included 2 × 5 min in Blotto and 3 × 5 min in Blotto without Carnation nonfat milk, at room temperature. Radiolabeled blots were dried briefly and exposed to Kodak X-AR 5 film for autoradiography at −70 °C.

RESULTS

Previous studies in our laboratory identified a group of low molecular weight proteins (14–20 kDa) having apparent nuclease activity by the Rosenthal and Lacks nuclease gel assay (31), that seem to be present in rat thymocyte nuclease extracts only after glucocorticoid treatment. Our attempts to purify this nuclease(s) were confounded by the copurification of histone H_2B, as confirmed by protein microsequencing. The presence of histone H_2B in this molecular weight range of lymphoid cell nuclear extracts was independently reported by Alnemri and Litwack (1). This data, combined with the observation that histone can block ethidium bromide binding to DNA (1, 35), which was the nuclease detection method on which we had previously relied, led several investigators to conclude that the nuclease(s) did not exist (1, 36, 46). Fig. 1 analyzes the nuclease activity exhibited by micrococcal nuclease, histone H_2B, and 0.4 M control and dexamethasone-treated thymocyte nuclear extracts using the modified nuclease assay with ^{32}P-radioactive DNA substrate. Micrococcal nuclease and both the control and dexamethasone extracts caused the generation of round clearings of the radioactive DNA, whereas the histone H_2B protein was unable to clear the gel of radioactive DNA. However, in contrast to our previous studies (3), as shown in Fig. 1, there is no difference in the amount of nuclease activity in the control and dexamethasone extracts. The difference between the present findings and our previous observations probably reflect several factors. First, extraction conditions used previously did clearly extract more histone proteins from dexamethasone-treated thymocytes (data not shown). Thus, we believe that the differences we observed previously reflected the assay which detected both histone and nuclease activity. Second, the modified assay now utilized is more sensitive and measures only nuclease activity. Thus it seems likely that our previous assay was not sufficiently sensitive to detect nuclease activity with the amounts of nuclear extract loaded on the gels. Finally, as shown in the H_2B panel of Fig. 1, some partial clearing around the histone protein is occasionally observed, whereas the center of the protein spot actually gets darker. This effect is independent of calcium and is probably caused by the ability of histones to bind and concentrate a small amount of DNA within their vicinity. Alternatively, this partial clearing may reflect some contamination of the histone H_2B preparation with a nuclease similar to NUC18. The fact that extensive boiling in SDS can abolish the partial clearing due to histone (data not shown) supports the latter explanation and suggests that the nuclease is distinct from H_2B. Based on these data, we concluded that the nuclease does exist and sought a technique that would separate the nuclease from histone H_2B.

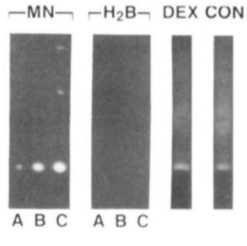

FIG. 1. **Nuclease activity assay comparing nuclease activity with histone H_2B.** The modified nuclease assay as described under "Materials and Methods" was used to analyze nuclease activity in three different protein preparations. Micrococcal nuclease (*MN*) lanes *A*, *B*, and *C* contain 0.1, 0.5, and 1.0 μg of micrococcal nuclease; H_2B lanes *A*, *B* and *C* contain 0.1, 0.5, and 1.0 μg of histone H_2B; and control (*con*) and dexamethasone (*dex*) lanes contain 2.5 μg of protein extracts.

As a first approach toward purifying the nuclease, the NaCl concentration of the extraction buffer was lowered from 0.6 M to 0.4 M in order to extract less histone proteins, and Bio-Rex 70 chromatography was subsequently used under conditions in which histones are known to have high affinity for the column matrix (38). Bio-Rex 70 chromatography in the presence of 0.4 M NaCl was sufficient to remove all detectable histone H_2B without appreciable loss of nuclease activity (Fig. 2). The removal of histone H_2B was confirmed by Western blot analysis. The *far left panel* shows a Coomassie stain of equal amounts of protein from crude extract (the 0.4 M NaCl dexamethasone extracts) and Bio-Rex 70 steps (dexamethasone extract after passage over Bio-Rex 70 in 0.4 M NaCl). This column separation resulted in the loss of approximately 15% of the total extract proteins. However, nuclease activity of the 18-kDa protein is not depleted, as shown in the *middle panel*, where crude extract and Bio-Rex 70 fractions have similar nuclease activities. Western blot analysis, shown in the *far right panel* of Fig. 2, confirms that, although histone H_2B is present in dexamethasone extracts, it is undetectable following Bio-Rex 70 chromatography. These data demonstrate that Bio-Rex 70 treatment effectively removes all detectable histone H_2B from dexamethasone extracts while retaining the nuclease activity associated with the 18-kDa protein.

Having efficiently separated NUC18 from histone H_2B, two additional chromatographic steps were used to purify this nuclease. We employed an anion exchange column, DEAE-Bio-Gel A, to which a majority of the nuclease did not bind

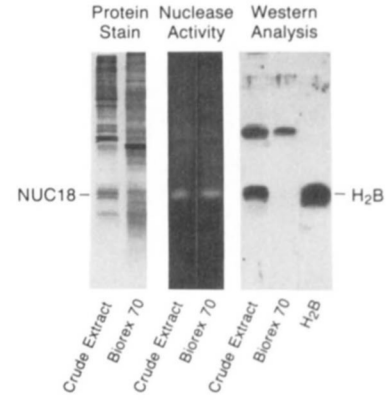

FIG. 2. **Bio-Rex 70 removes histone H_2B.** *Lanes 1* and *2* in each *panel* contain equal amounts of the 0.4 M NaCl dexamethasone extract (*Crude Extract*) and the 0.4 M NaCl Bio-Rex 70 fraction. The *left panel* depicts a Coomassie Brilliant Blue-stained gel containing 10 μg of each extract, the *middle panel* represents an autoradiographic film of the modified nuclease assay of 5 μg of each protein extract, and the *right panel* shows a Western blot of 20 μg of each extract or 2 μg of H_2B hybridized with anti-histone H_2B antibody (generously blot supplied by Dr. Chi-Bom Chae, University of North Carolina).

in the presence of 10 mM NaCl. Under these conditions, 82.5% of the total extract proteins bound to this column, leaving only 2.5% after the first two columns (15% was removed by the Bio-Rex 70 column, and 82.5% was removed by DEAE chromatography). The remaining protein was bound to the hydrophobic interaction column, phenyl-Sepharose, in the presence of 1.5 M NaCl, and NUC18 nuclease activity was eluted with 0.5 M NaCl. This step allowed concentration of the sample from over 250 ml down to 20 ml while removing an additional 0.5% of the total protein. Fig. 3 shows a silver-stained gel of samples obtained from this purification scheme. The first four lanes contain equal quantities of the protein from each purification step. Following Bio-Rex 70 chromatography, loss of the negatively staining low molecular weight histones is apparent, and in the phenyl-Sepharose lane, a protein of 18 kDa has been enriched. The phenyl-Sepharose fraction was further concentrated and subjected to preparative SDS-PAGE and electroblotting as described under "Materials and Methods." The eluted NUC18 protein is shown in the SDS-PAGE lane of Fig. 3. The protein appears homogeneous by this criterion; however, it is impossible to preclude co-migration with other proteins by this technique.

Following purification by this scheme, NUC18 protein retains biological activity, as shown in Fig. 4. The second lane in Fig. 4 is an autoradiographic exposure of the nuclease activity gel assay demonstrating the calcium-dependent nuclease activity of purified NUC18. The amount of protein in the nuclease activity lane represents approximately one-fifth the amount of protein shown silver-stained in the first lane of Fig. 4. The last two lanes in Fig. 4 show a Western analysis of equal quantities of NUC18 and histone H_2B hybridized with a second anti-histone H_2B antiserum. The NUC18 lane contains no detectable histone H_2B, whereas the H_2B lane strongly reacts with the antiserum. Thus, we have purified a biologically active NUC18 protein that is immunologically distinct from histone H_2B.

We next sought to characterize the enzymatic requirements of this protein and to compare them with other known nucleases. We used the nuclease activity gel system to characterize the pH and ionic requirements of NUC18. NUC18 requires a neutral pH, having optimal activity at pH 7.0–8.5 (data not shown). Fig. 5 shows results from representative gels of several more of these experiments. Each panel contains three lanes that correspond to micrococcal nuclease (0.2 μg) and mammalian DNase I (1 μg) as the two positive nuclease controls and NUC18 (0.2 μg of purified NUC18). The *first panel* demonstrates that, in the absence of calcium, all three enzymes are inactive. The *second panel* shows that, in the presence of 2 mM calcium, all three enzymes, micrococcal nuclease, DNase I, and NUC18, are activated. The *third panel* demonstrates that a 1-h pretreatment with 2 mM zinc before addition of 2 mM $CaCl_2$ was sufficient to inhibit nuclease activity of NUC18. This concurs with a report of Cohen and Duke (28) in which internucleosomal DNA cleavage could be prevented by treatment with zinc. In the *fourth panel*, a 1-h pretreatment with 1 mM aurintricarboxylic acid prevents calcium-dependent activity of NUC18, and micrococcal nuclease (*MN*). Aurintricarboxylic acid is a nonspecific nuclease inhibitor (45) and has been reported to block internucleosomal DNA cleavage and other properties of apoptosis in thymocytes (41). The *last panel* demonstrates that, in the presence of 2 mM manganese and no calcium, both micrococcal nuclease and DNase I are activated while NUC18 remains unactivated.

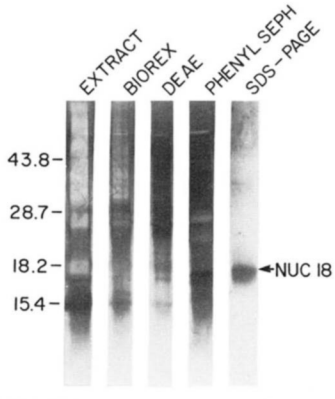

FIG. 3. **SDS-PAGE analysis of NUC18 purification.** Aliquots (10 μg) of fractions after each purification step were electrophoresed in 15% SDS-polyacrylamide gels and subsequently silver-stained as described under "Materials and Methods." *Lane 1*, 0.4 M NaCl dexamethasone extract; *lane 2*, 0.4 M NaCl Bio-Rex 70 eluant; *lane 3*, 10 mM NaCl DEAE eluant; *lane 4*, 0.5 M NaCl phenyl-Sepharose fraction; *lane 5*, approximately 1.5 μg of NUC18 after transfer and elution following SDS-PAGE.

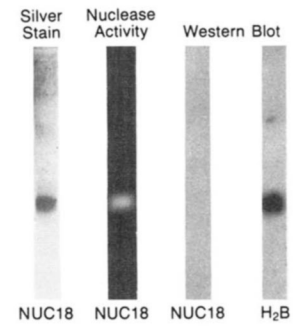

FIG. 4. **Nuclease activity and Western blot of purified NUC18.** Using the modified nuclease activity assay described in detail under "Materials and Methods," nuclease activity appears as a white hole on a dark radiolabeled DNA background. NUC18 purified as described in the text results in enzymatically active nuclease as shown here. On the *left* is a silver stain of approximately 1.5 μg NUC18, and on the *right* is a nuclease gel assay of approximately 0.3 μg of NUC18 protein. Western analysis was performed using an antibody to histone H_2B (graciously supplied by Michael Bustin, National Cancer Institute, NIH) and developed with ^{125}I-labeled protein A as described under "Materials and Methods." The *left lane* contains approximately 2 μg of purified NUC18, and the *right lane* contains 2 μg of purified histone H_2B.

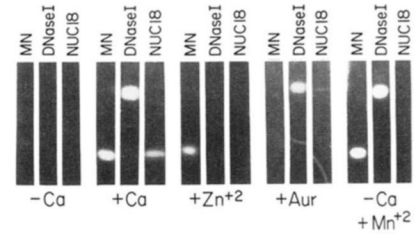

FIG. 5. **Characterization of purified nuclease activity.** Purified nucleases were analyzed by the modified nuclease gel assay described under "Materials and Methods." Following electrophoresis, gels were washed overnight to remove SDS and then soaked in TEM Buffer containing either 2 mM $MgCl_2$, $MnCl_2$ or $ZnCl_2$, or 1 mM aurintricarboxylic acid for 1 h prior to the addition of calcium. Gels were then incubated in the presence of 2 mM $CaCl_2$ for 3 h at 37 °C. *Lane 1* in each *panel* contains 0.25 μg of micrococcal nuclease (*MN*), *lane 2* contains 0.20 μg of pancreatic DNAse I, and *lane 3* contains approximately 0.20 μg of NUC18. The *first panel* depicts a gel in the absence of calcium, and the *next panel* shows nuclease activity in the presence of 2 mM calcium, demonstrating that all three enzymes are in fact calcium-dependent. *Panel 3*, the gel was incubated in TEM buffer plus 2 mM zinc plus calcium; *panel 4*, TEM buffer plus 1 mM aurintricarboxylic acid plus calcium; in *panel 5*, TE buffer (TEM without the $MgCl_2$) plus 2 mM $MnCl_2$ in the absence of calcium.

This result suggests that NUC18 is probably not related to mammalian DNase that can be activated by manganese.

Having proven the existence of an 18-kDa nuclease with unique enzymatic requirements, we next wished to re-examine the regulation of the NUC18 protein by glucocorticoids since studies from other laboratories (14, 24), as well as our own data presented in Fig. 1 suggested that it is also present in control cells. We evaluated the presence of NUC18 in control and dexamethasone extracts further by using gel filtration chromatography, which allows the determination of the molecular weight of the NUC18 protein under nondenaturing conditions. SDS-PAGE nuclease activity gel analysis of the column fractions is depicted in Fig. 6. The *far right lane* in the *upper* and *lower panels* demonstrates similar nuclease activity in extracts obtained from control and dexamethasone-treated cells. Clearing of radioactive DNA through the upper portion of the lanes may reflect trace deposits of the enzyme during electrophoresis, since more protein was loaded in these samples than in previous figures. The *lower panel* demonstrates that NUC18 from dexamethasone extracts elutes at two different molecular weights during gel filtration; one peak elutes at approximately 25 kDa and the other peak elutes in the void volume (*VV*), having a molecular mass of greater than 100 kDa. Interestingly, NUC18, as detected by SDS nuclease activity gels, is present only in the void volume after gel filtration of the control extracts (the 25-kDa form is absent under the nondenaturing gel filtration conditions). These data suggest that NUC18 can exist in two forms; one is perhaps bound to other proteins or to DNA and exists as a high molecular weight complex, and the second form exists at a molecular weight very close to its apparent molecular weight by SDS-PAGE. Since control extracts contain only the high molecular weight form, we conclude that dexamethasone treatment stimulates either synthesis of NUC18 or release of the smaller molecular weight form from the high molecular weight complex. The latter mechanism would explain how the nuclease in the untreated thymocytes could be present without generating measurable DNA degradation, whereas the dexamethasone-treated thymocyte extensively degrades its DNA.

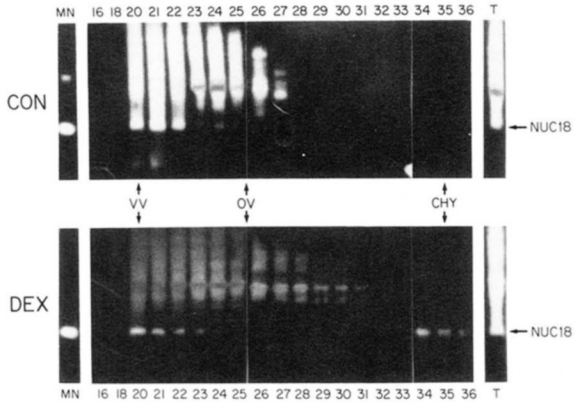

FIG. 6. **Bio-Gel P-100 elution profile of control and dexamethasone extract.** 5 mg each of control (*con*) (*upper panel*) and dexamethasone (*dex*) (*lower panel*) 0.4 M NaCl extracts were fractionated on a 75-ml Bio-Gel P-100 column in the presence of 0.4 M NaCl, 20 mM Tris, pH 7.8, and 1 mM EDTA. 1.5-ml fractions were collected, and a 20-μl aliquot from each fraction was analyzed by the modified nuclease assay. Molecular weight markers are as follows: blue dextran for the void volume (*VV*); ovalbumin (*OV*), 43,000; chymotrypsinogen (*CHY*), 25,000. *Lane 1* in each *panel* contains 0.25 μg of micrococcal nuclease (*MN*), and the *last lane* (*T*) in each panel contains 5 μg total extract (*T*) of either control or dexamethasone 0.4 M NaCl extract. *Lanes 2–20* contain sequential 20-μl aliquots of column fractions from the Bio-Gel P-100 column.

These mechanisms would also be consistent with the literature and our previous data demonstrating that inhibition of protein synthesis blocks glucocorticoid-induced lymphocytolysis and suggest that synthesis of some protein, perhaps an activator protein or the nuclease itself, is required for DNA degradation to occur (25, 29, 30).

DISCUSSION

We have purified an 18-kDa nuclease (NUC18) from dexamethasone-treated rat thymocytes using the combined approaches of ion exchange chromatography, hydrophobic interaction chromatography, and preparative SDS-PAGE. NUC18 has calcium-dependent nuclease activity by the nuclease activity gel assay. Our previous purification strategy resulted in a preparation of NUC18 that was contaminated with histone H_2B, as confirmed by protein microsequencing. However, as demonstrated by Western blot analysis, this new purification scheme results in purified NUC18 that is distinct from histone H_2B. These findings refute three recent reports (1, 36, 46) that suggest that the 15–20 kDa endonuclease activity in lymphoid cells is due to histone H_2B. These authors concluded that the "nuclease activity" we reported was due to the ability of histones to quench the fluorescence of ethidium bromide-stained DNA. However, Rosenthal and Lacks (35), who initially developed this nuclease assay, subsequently demonstrated that, if radioactive DNA is incorporated into the gel, only nuclease activity will be detected, since histones do not block the radioactive emissions from ^{32}P. Further, they (35) crucially demonstrated that detection of histones by ethidium bromide staining takes place in the absence of added divalent cations and, in fact, may be inhibited by the presence of cations. The data presented in this manuscript are consistent with these criteria for discerning nuclease activity from DNA-binding activity, since NUC18 is calcium-dependent in these activity gels. We believe that the discrepancy between our studies and Alnemri and Litwack involving CEM-C7 cells reflect their use of inappropriate cells as a source of nuclei for nuclease purification. It is well recognized that CEM-C7 cells do not respond to glucocorticoids by the same mechanisms as do rat thymocytes and, therefore, it is likely that these cells may not express the appropriate nuclease. In fact, following their initial report, Alnemri and Litwack have subsequently published that CEM-C7 cells do not contain a calcium-dependent endonuclease (32). This observation readily explains why these investigators were unable to purify calcium-dependent nuclease activity from nuclear extracts of CEM-C7 cells.

In the present study, we have used a modified nuclease activity gel system to characterize the nuclease activity of the purified NUC18 protein. The nuclease requires a neutral pH and is calcium-dependent. NUC18 is a basic protein, having a pI of 8.5 or greater as determined by chromatofocusing (data not shown). In addition, NUC18 is probably not related to DNase I since DNase I but not NUC18 is stimulated by manganese alone. Many reports in the literature refer to a calcium- and magnesium-dependent endonuclease (14, 28, 37, 38) in thymocytes; however, the requirement of NUC18 for magnesium can not be rigorously tested in our studies because high concentrations of magnesium are present during the extraction procedure. NUC18 is inhibited by both zinc and a general nuclease inhibitor, aurintricarboxylic acid. Zinc treatment of thymocytes *in vitro* has been shown to prevent internucleosomal DNA degradation, as well as thymocyte cell death (28). In addition, zinc-deficient mice have smaller than normal thymi, whereas the thymus remains normal in size if the zinc-deficient mice have been previously adrenalectomized

(39, 40). These results suggest that zinc may exert a protective effect against glucocorticoid-induced lymphocytolysis in the thymus. Aurintricarboxylic acid has also been shown to block internucleosomal DNA cleavage and apoptosis (41). The enzymatic characteristics of NUC18, as well as its sensitivity to specific inhibitors known to block internucleosomal DNA cleavage in lymphoid cells, strongly support the hypothesis that NUC18 plays an important role in glucocorticoid-induced DNA degradation integral to the apoptotic process.

NUC18 is present in extracts from both control and dexamethasone-treated thymocytes in a high molecular weight complex (>100 kDa), as determined by gel filtration analysis. However, dexamethasone extracts differ from controls in that they also contain NUC18 in fractions that correspond to a molecular weight of approximately 25 kDa. There are several possible explanations for the occurrence of this 25 kDa nuclease activity in dexamethasone-treated thymocytes and its absence in control extracts. These possibilities include differential extraction of proteins in the two extracts due to the presence of degraded DNA in the dexamethasone extracts; induction of enzyme activity through transcriptional activation via the classical steroid receptor pathway; specific nuclease mRNA stabilization; and activation via nongenomic mechanisms, perhaps due to availability of cofactors or to conversion from an inactive precursor into an active nuclease. We do not believe that differential extraction explains our data since the 25-kDa form is apparently not associated with DNA, as it does not bind to DEAE-Bio-Gel A, whereas the high molecular weight form does bind to the DEAE column and exactly coelutes with the DNA (data not shown). Transcriptional activation remains a possible mechanism, since genetic evidence exists that indicates that cell death in response to steroid hormone is mediated by a specific gene product or "lysis" gene (42). The exact role for this lysis gene is unclear at this time. Its product may be responsible for causing an influx of calcium that is required since the enzyme is calcium-dependent, it may induce another protein responsible for initiating a cascade of events that lead to activation of internucleosomal cleavage activity, or it may actually be involved in the internucleosomal cleavage itself.

Based on the ubiquitous observation that internucleosomal DNA degradation of DNA occurs in all reported instances of apoptosis, we hypothesize that this DNA degradation is the first irreversible process in programmed cell death. The correlation between detection of this low molecular weight nuclease and DNA degradation makes this protein a strong candidate for the apoptotic nuclease.

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